2344–2352 *Nucleic Acids Research, 1998, Vol. 26, No. 10*

A differential response of wild type and mutant promoters to TFIIIB$_{70}$ overexpression *in vivo* and *in vitro*

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Received February 3, 1998; Revised and Accepted March 30, 1998

ABSTRACT

TFIIIB, the initiation factor for transcription by RNA polymerase III (pol III) is, in yeast, composed of three subunits: TBP, TFIIIB$_{70}$/Brf1 and TFIIIB$_{90}$. To determine the extent to which each of these subunits is limiting for pol III transcription, the effect of overexpressing each subunit was assessed on the expression of wild-type and promoter mutant pol III genes both *in vivo* and *in vitro*. *In vivo*, we find that the synthesis of wild-type pol III genes is not limited to a significant extent by the level of any TFIIIB subunit. There is, however, a two-fold increase in the synthesis of the promoter mutant gene, *sup9-e A19-supS1*, in strains overexpressing TFIIIB$_{70}$. The findings suggest that overexpression of TFIIIB$_{70}$ has a differential effect on the expression of pol III genes with strong versus weak promoters. *In vitro* transcription assays support this conclusion and reveal an inverse correlation between the transcriptional response to TFIIIB$_{70}$ overexpression and promoter strength. The individual TFIIIB subunits are nuclear by immunofluorescence and are calculated to have nuclear concentrations in the low micromolar range. In comparison, the factors are diluted 100-fold or more in whole cell extracts. This dilution accounts for the generally limiting nature of TFIIIB$_{70}$ in pol III gene transcription *in vitro.*

INTRODUCTION

RNA polymerase III (pol III) transcription in the yeast *Saccharomyces cerevisiae* requires three factors, TFIIIA, TFIIIB and TFIIIC (1–3). TFIIIB serves as the initiation factor in this system and is assembled upstream of the transcription start site by TFIIIA and TFIIIC in the case of 5S genes or by TFIIIC alone in the case of other pol III genes (4). *In vivo*, TFIIIC and TFIIIA are required for the efficient assembly of pol III-specific complexes (5,6). However, *in vitro* TFIIIB can be assembled on some DNAs in the absence of these factors. This reaction is initiated by the sequence-specific binding of one of its three subunits, namely the TATA binding protein (TBP) to templates containing a TATA box (7–9). The remaining two components, a 70 kDa polypeptide (TFIIIB$_{70}$) with homology to TFIIB, and a protein termed B″ or TFIIIB$_{90}$ which has an apparent molecular mass of 90 kDa, can then associate with this complex (9–12). TFIIIB–DNA complexes assembled in this manner or via TFIIIA and/or TFIIIC are extraordinarily resistant to high salt concentrations and to polyanions and have the ability to direct multiple rounds of initiation by the polymerase (4,7,9). These properties have so far only been demonstrated for yeast TFIIIB–DNA complexes. However, similar properties are anticipated in higher eukaryotes based on recent findings which demonstrate that human TFIIIB includes proteins that are structurally and/or functionally homologous to the yeast subunits (13–16).

In the absence of DNA, the subunits of yeast TFIIIB are thought to be rather loosely associated. For example, TBP and TFIIIB$_{70}$ can be separated from TFIIIB$_{90}$ by chromatography on Mono S resin (17) while TBP and TFIIIB$_{70}$ are readily separated from one another using tagged forms of the latter protein (18). These results contrast with those from another study which showed that TFIIIB can be immunopurified using antibodies against TBP (19). Several explanations have been proposed to account for this apparent difference in complex stability (18). However, the central questions about the nature of TFIIIB in the cell and whether it is recruited to the DNA in a single step or in multiple steps have not been resolved. With the availability of all three subunits of yeast TFIIIB in recombinant form, it has become possible to reexamine these issues and to address other qualitative and quantitative aspects of TFIIIB assembly and function (for example see 11,12,20).

In a previous study we reported that multiple copies of the wild-type gene encoding TFIIIB$_{70}$ suppressed the transcriptional defect associated with a tRNA gene A block promoter mutation (21). This result suggested that TFIIIB$_{70}$ was limiting for pol III transcription in yeast. Subsequently, multi-copy suppression of conditional mutations in TFIIIC subunits was demonstrated by TFIIIB$_{70}$ and by the other two subunits of TFIIIB suggesting that the latter polypeptides were also limiting under these conditions (22). In addition, studies in *Drosophila* Schneider S-2 cells and rat 1A fibroblasts have shown that pol III transcription is limited by the amount of TBP (23,24). In the *Drosophila* system, this effect was shown to be direct since the increase could be

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prevented by pol III-specific mutations in TBP. In light of these findings, we have examined the effect of increasing the gene dosage of each subunit of yeast TFIIIB on pol III transcription directed by both wild-type and mutant promoters. In addition, we have determined the cellular and extract levels of the TFIIIB components in order to correlate the results of our functional studies with emerging biochemical data on subunit–subunit interactions.

MATERIALS AND METHODS

Plasmids

The genes encoding the yeast factors TBP, $TFIIIB_{70}$ and $TFIIIB_{90}$ were cloned into the 2μ plasmid vector pRS423 which carries the *HIS3* gene as a selectable marker. The gene encoding TBP was recloned from pDE28-6 (provided by Greg Prelich) as a 2.4 kb *Sac*I/*Sal*I fragment to give p423TBP. The gene encoding $TFIIIB_{90}$ was recovered by PCR amplification from a wild-type genomic bank using the primers 5′-CCGTAAGAGCTCCAAA-CTCAAGGC-3′ and 5′-CTCTGGACAGCTGTAGTCCAC-3′. A 3.3 kb *Sac*I/*Pvu*II-cleaved PCR product was cloned into *Sac*I/*Sma*I-cleaved pRS313 and the insert was subsequently transferred into pRS423 to give p423B90. The 2.6 kb insert from pRSM3 (21) which contains $TFIIIB_{70}$ was recloned into pRS423 to give p423B70. The multi-copy plasmid pUKC352 (obtained from Mick Tuite) contains the strong *PGK1* promoter and the first 33 N-terminal amino acids from the *PGK1* gene fused to a 21 bp oligonucleotide which includes an in-frame UAG stop codon followed by the *lacZ* gene (amino acids 8–1021). The wild-type *sup9-e* gene and promoter mutants in either *sup9-e* or the homologous *sup3-e* gene were all cloned in the plasmid YCp50 (25).

Yeast strains and phenotype analysis

The strains IW1B6 (26) and YSB108 (27) were used for the preparation of whole cell extracts. Phenotypic analysis of TFIIIB subunit overexpression was conducted in strains UB6 and UB7. Strain UB6 was constructed by integrating a single copy of the dimeric *sup9-e A19-supS1* gene at the *URA3* locus of strain IW1B6 as described previously (26). Strain UB7 was constructed in the same way but contains a single copy of the monomeric *supS1* gene. The suppressor phenotypes of strains overexpressing the individual TFIIIB subunits were examined by growing saturated cultures in synthetic complete media lacking uracil and histidine (to select for both the plasmid and the suppressor). The cells were washed, normalized for cell concentration, serially diluted and spotted onto minimal media plates containing leucine. Growth on this medium requires the expression of amber nonsense suppressor activity and maintains selection for the plasmid and the suppressor.

In vitro transcription

Whole cell extracts were prepared in parallel as previously described (26) from 8–10 g of cells grown to an absorbance at 600 nm of ~3.0. Transcription reactions were carried out in 50 μl reactions containing 1 μg of template DNA as described (26). Assays were performed under multiple round conditions at 15°C for 60 min. The RNA products were analyzed on denaturing 8% polyacrylamide gels and visualized by autoradiography or with phosphor storage plates and a phosphorimager (Molecular Dynamics). The amount of RNA was determined by excising the bands and measuring the incorporation of [α-^{32}P]GTP by Cerenkov counting or by image analysis with ImageQuaNT software (Molecular Dynamics).

β-galactosidase assays

β-Galactosidase activity was measured in transformants of strain UB6 containing an amber suppressible *PGK1-lacZ* reporter gene on the plasmid pUKC352 (28) and either pRS423 or derivatives containing the individual TFIIIB subunits. Cells were grown in synthetic complete medium lacking uracil, leucine and histidine which selects for the suppressor, the TFIIIB subunit overexpressing plasmid and the *lacZ* reporter plasmid. Assays were conducted on two to four samples for each strain in each experiment as described (29). Standard deviations and standard errors were propagated according to Bevington (30).

Antibodies and western analysis

Yeast cells in log phase were lysed by glass bead breakage in a mini-beadbeater (Biospec) into RIPA buffer containing protease inhibitors as described (31). Breakage was monitored by light microscopy. Antibodies to $TFIIIB_{70}$ have been described previously (32). Rabbit polyclonal antibodies were raised against recombinant yeast TBP purified as described (33). The specificity of this antibody was demonstrated by its recognition of a 27 kDa band in yeast nuclear and whole cell extracts, in rabbit reticulocyte lysates containing *in vitro* synthesized TBP and in *Escherichia coli* extracts induced for the expression of recombinant TBP (34). Rabbit polyclonal antibodies were raised against recombinant yeast $TFIIIB_{90}$ purified by preparative SDS–polyacrylamide gel electrophoresis as described for $TFIIIB_{70}$ (11). Similar criteria as described above for TBP were used to demonstrate the specificity of this antibody for $TFIIIB_{90}$. Western analysis was performed as described previously (32) using a 1:5000 dilution of all primary and secondary antibodies. Efficient detection of $TFIIIB_{90}$ required the presence of 0.4% SDS in the transfer buffer and a 12 h incubation with primary antibody. Antibody–antigen complexes were detected by enhanced chemiluminescence (Amersham Corp) and quantified using laser densitometry and ImageQuaNT software (Molecular Dynamics). The amounts of each recombinant standard are based on measurements of intrinsic fluorescence at 280 nm. Standard curves for individual TFIIIB subunits were normalized using the slopes determined by linear regression. The high precision of the ECL quantification is reflected by the coefficients of variation of the slopes of the bestfit lines generated in an analysis of six standard curves. For TBP and $TFIIIB_{70}$ the coefficients of variation are 6.1% and 3.5%, respectively.

Northern analysis

Total RNA was extracted from transformants of strain UB6 containing either pRS423 or p423B70 by glass bead disruption in the presence of hot phenol (35). The RNA (5–40 μg per lane) was resolved on 10% polyacrylamide, 8.3 M urea gels in TBE buffer. After electrophoresis, gels were soaked for 10 min in 0.5× TBE prior to electrophoretic transfer (0.5× TBE and 100 V for 2 h at 4°C) of the RNA to Zetaprobe nylon membrane (BioRad) using a BioRad Transfer apparatus. After transfer, the membranes were dried for 10 min and the RNA was crosslinked by UV irradiation (0.2 J/cm). For northern analysis of pre-*RPR1* RNA, electrophoresis was performed on a 1.2% agarose formaldehyde gel. In this case,

capillary transfer of the RNA to a nitrocellulose membrane was performed using 20× SSC. Pre-hybridizations and hybridizations were carried out overnight in 6× SSPE, 0.1% SDS, 2× Denhardt's and 125 μg/ml denatured salmon sperm DNA at 37°C. The oligonucleotide probes 5′-CCAAACAACCACTTATTTGTT-GA-3′ and 5′-GTGCCATTTCGATTTGAAA-3′ were used to detect 5′ leader-containing pre-tRNA$^{Leu}_3$ (CAA, 132 nt) and pre-tRNA^{Ser} (CGA, 123 nt), respectively and were provided by Craig Peebles. The oligonucleotides 5′-TCGGACATAGTGACT-TCTT-3′ and 5′-CAGCCGCGAGCACCACAGCGT-3′ were used to detect the 5′ leader-containing precursors of *sup9-e A19* (200 and 108 nt precursors correspond to the dimeric *sup9-e A19-supS1*tRNA precursor and the monomeric *sup9-e A19* precursor lacking *supS1*) and *RPR1* (486 nt), respectively. U4 RNA (160 nt) was detected using the probe 5′-CCATGAGGAGACGGTCTGG-3′. The oligonucleotides (20 pmol) were end-labeled in 40 μl reactions using [γ-^{32}P]ATP 300 μCi, 3000 Ci/mmol (Amersham Life Science) and T4 polynucleotide kinase. Unincorporated nucleotides were removed using Sephadex G-25 spin columns. The final posthybridization washes were in 6× SSPE 0.1% SDS at 37°C. Hybridization was detected using phosphor storage plates and a phosphorimager (Molecular Dynamics). RNA levels were normalized using the signal for U4 RNA. Relative RNA levels were determined by linear regression of data obtained from two or more independent blots.

Immunofluoresence

In situ hybridization was performed as described previously (36,37) except that the spheroplast buffer (1.2 M sorbitol, 100 mM potassium phosphate pH 7.5) did not contain RNase inhibitors. A 1:250 dilution of the antibodies in blocking buffer (10 mg/ml BSA in phosphate-buffered saline, 0.1% NaN_3) was incubated with the spheroplasts overnight at 4°C. The cells were subsequently washed with blocking buffer and incubated for 2 h with Cy3-conjugated anti-rabbit antibody at a 1:2000 dilution. Fluorescent images were acquired with a Photometrics PXL cooled CCD camera (Tuscon, AZ) on an Olympus IX-70 microscope with 60× N.A. 1.4 planapo optics. Fluorescent filter sets were narrow band pass to eliminate spillover from one channel to any other. Images were prepared for publication using NIH-Image and Adobe Photoshop.

RESULTS

Identification of the limiting TFIIIB subunit *in vivo*

To examine the extent to which the different subunits of TFIIIB are limiting for pol III transcription *in vivo*, multi-copy plasmids containing TBP, TFIIIB$_{70}$ and TFIIIB$_{90}$ were transformed into a strain harboring the reporter construct *sup9-e A19-supS1*. The expression of *supS1* amber nonsense suppressor activity in these cells serves to report on transcription directed by the mutant (A19) promoter of the *sup9-e* gene (26 and Fig. 1). TFIIIB$_{70}$ appears to be the only TFIIIB subunit that can, when overexpressed, increase the suppressor activity of *supS1* in this dimeric context. The growth of strains which overexpress (see below) either TBP or TFIIIB$_{90}$ is comparable to the control strain. Western analysis of whole cell lysates prepared under denaturing conditions (see Materials and Methods) showed that strains overexpressing TFIIIB$_{70}$, TBP or TFIIIB$_{90}$ contained respectively, 22-fold, 10-fold or 7-fold more of the corresponding protein than the control (data not shown).

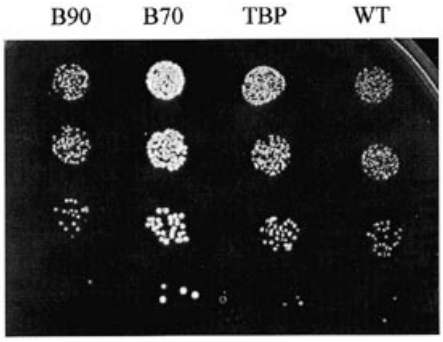

Figure 1. Amber suppressor phenotype of *S.cerevisiae* strain UB6 overexpressing individual TFIIIB subunits. Strain UB6 which contains an integrated copy of the *sup9-e A19-supS1* gene was transformed with the plasmid pRS423 (WT) or derivatives bearing the genes encoding TBP, TFIIIB$_{70}$ (B70) or TFIIIB$_{90}$ (B90). Transformants were grown to saturation in synthetic complete medium lacking uracil and histidine and were normalized for cell concentration before being serially diluted and spotted onto a minimal medium plate. Growth on this medium selects for the suppressor locus (Ura+), the plasmid (His+) and expression of *supS1* amber suppressor activity (Trp+, Met+). The cells were photographed after 72 h of incubation at 30°C.

The above suppression assay provides only a qualitative measure of *sup9-e A19-supS1* function. We therefore employed a quantitative termination codon readthrough assay to determine *supS1* suppressor activity (28). Strains containing each TFIIIB subunit on a multicopy plasmid (Fig. 1) were transformed with the plasmid pUKC352 which carries an amber-suppressible *lacZ* reporter construct. The results of β-galactosidase assays conducted over four separate experiments are shown in Table 1. In good agreement with the phenotypic assay, the TBP overexpressing strain showed only a slight increase (1.2-fold) in β-galactosidase activity while the TFIIIB$_{90}$ overproducer showed no significant change in activity relative to the wild-type strain. In contrast, overexpression of TFIIIB$_{70}$ resulted in a 2-fold increase in β-galactosidase activity. This result establishes that TFIIIB$_{70}$ but not TBP or TFIIIB$_{90}$, is the principal limiting component of TFIIIB in the expression of *sup9-e A19-supS1 in vivo*.

Table 1. Quantitation of *supS1* suppressor activity in strains overexpressing individual TFIIIB subunits

Overexpressed TFIIIB subunit	TBP	TFIIIB$_{70}$	TFIIIB$_{90}$
Expt 1[a]	1.1 ± 0.13	1.9 ± 0.13	0.8 ± 0.07
Expt 2[a]	1.1 ± 0.05	2.0 ± 0.24	0.9 ± 0.06
Expt 3[a]	1.3 ± 0.20	1.9 ± 0.12	0.9 ± 0.17
Expt 4[a]	1.3 ± 0.23	2.2 ± 0.46	1.2 ± 0.26
Average[b]	1.2 ± 0.04	2.0 ± 0.06	1.0 ± 0.04

[a]Fold difference and standard deviation in β-galactosidase activity relative to the wild-type strain.
[b]Average fold difference and standard error in β-galactosidase activity relative to the wild-type strain.

A quantitative difference between *in vivo* and *in vitro* assays of TFIIIB$_{70}$ overexpression

Previous studies have indicated that elevated levels of TFIIIB$_{70}$ can increase transcription *in vitro* to a greater extent than the

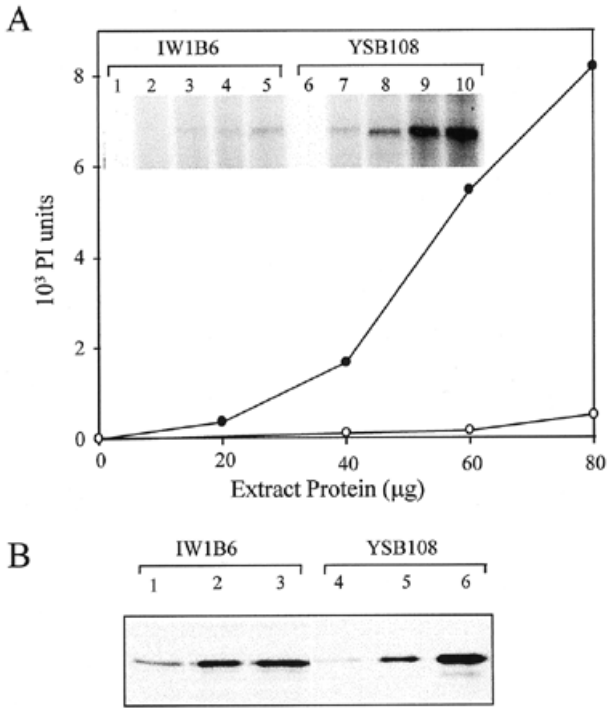

Figure 2. Overexpression of $TFIIIB_{70}$ increases transcription *in vitro*. (**A**) The protein concentration dependence of transcription in whole cell extracts prepared from wild-type (IW1B6) and $TFIIIB_{70}$-overexpressing (YSB108) strains. The inset shows the primary dimeric transcript synthesized under multiple-round conditions using the *sup9-e A19-supS1* gene (1 µg) as a template. Reactions in lanes 1–5 and lanes 6–10 contained 0, 20, 40, 60 and 80 µg of extract protein, respectively. The plot presents the data obtained by phosphorimage analysis of the inset. A 17-fold difference in transcription was calculated from the linear portion of the curves. (**B**) Western analysis of IW1B6 and YSB108 whole cell extracts detected for $TFIIIB_{70}$. Lanes 1–3 contain 5.0, 10.0 and 15.0 µg of protein from the IW1B6 extract and lanes 4–6 contain 0.8, 1.6 and 3.2 µg of protein from the YSB108 extract, respectively.

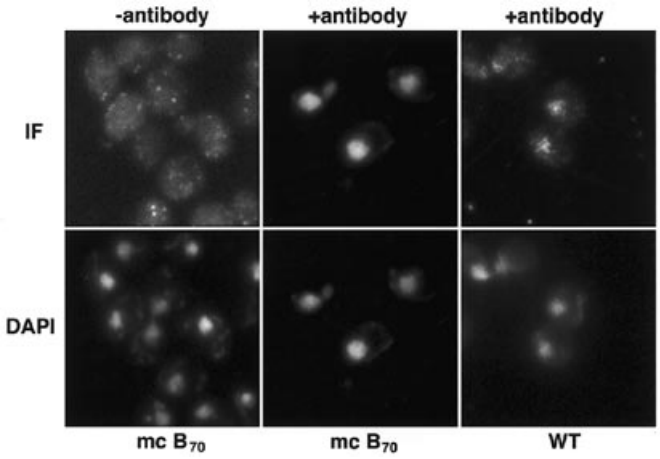

Figure 3. Localization of $TFIIIB_{70}$ by immunofluoresence. Fixed and permeabilized wild-type (WT) and $TFIIIB_{70}$ overexpressing cells (mc B_{70}) were incubated with or without antibodies to $TFIIIB_{70}$ (1:250 dilution) or with DAPI stain. Anti-rabbit secondary antibodies were conjugated with CY3.

2-fold effect observed *in vivo* (21,32,38). However, a direct quantitative comparison of $TFIIIB_{70}$ overexpression and its effect on transcription *in vivo* and *in vitro* has not been described. We therefore prepared whole cell extracts from a control strain (IW1B6) and a strain (YSB108) whose viability required the maintenance of a multicopy plasmid containing $TFIIIB_{70}$. Figure 2A shows the protein concentration dependence of transcription in these extracts. In the linear portion of the curves, the YSB108 extract is 17-fold more active in transcription of the *sup9-e A19-supS1* gene than the control extract. This is significantly greater than the 2-fold increase in the expression of this gene *in vivo* (Table 1). To examine the possibility that the large *in vitro* effect results from differential extraction of $TFIIIB_{70}$, the relative amount of the protein in these extracts was determined by quantitative western blot analysis (see Materials and Methods and Fig. 2B). Densitometry of the western blot revealed that the YSB108 extract contained ~20 times the amount of $TFIIIB_{70}$ that was present in the control extract. This is in good agreement with the previously determined 22-fold increase observed in whole cell lysates prepared under denaturing conditions (cited above). The close correspondence between the level of $TFIIIB_{70}$ overexpression and the increase in transcription *in vitro* indicates that this factor is at least an order of magnitude more limiting than the next most limiting factor in the extract.

$TFIIIB_{70}$ is localized to the nucleus

The quantitative difference between the *in vivo* and *in vitro* assays concerning the effect of $TFIIIB_{70}$ overexpression may be explained in several ways. It is possible that *in vivo* $TFIIIB_{70}$ is not confined to the nucleus. $TFIIIB_{70}$ localized in cellular compartments other than the nucleus would not affect pol III transcription *in vivo*. However, the disruption of cellular barriers with the preparation of whole cell extracts would enable this material to participate in the assembly of transcription complexes. We therefore examined the localization of $TFIIIB_{70}$ in control and overexpressing cells by immunofluoresence. Figure 3 shows representative fields of these cells after treatment with or without an anti-$TFIIIB_{70}$ polyclonal antibody or DAPI stain. A specific, well-defined signal for $TFIIIB_{70}$ can be seen in both strains treated with the primary antibody. As expected, the signal was significantly more intense in the overexpression strain. More importantly however, DAPI staining of the chromosomes confirmed the nuclear localization of $TFIIIB_{70}$ in both cases. There was no detectable cytoplasmic staining for $TFIIIB_{70}$ in either strain. In parallel experiments, the nuclear localization of TBP and $TFIIIB_{90}$ in wild-type and overexpressing strains was also confirmed (data not shown). These results show that (i) overexpression of the TFIIIB subunits, up to 20-fold in the case of $TFIIIB_{70}$, does not affect their subcellular localization and (ii) the amount of each TFIIIB subunit in a cell closely approximates the nuclear level of the protein.

A correlation between promoter strength and limiting levels of $TFIIIB_{70}$

Another possible explanation for the quantitative difference observed *in vivo* and *in vitro* upon $TFIIIB_{70}$ overexpression is that the increased synthesis of *sup9-e A19-supS1 in vivo* may be offset, to a large extent, by an increase in RNA turnover. In this way, the steady state level of mature *supS1* tRNA could be maintained at close to wild-type levels, effectively limiting the

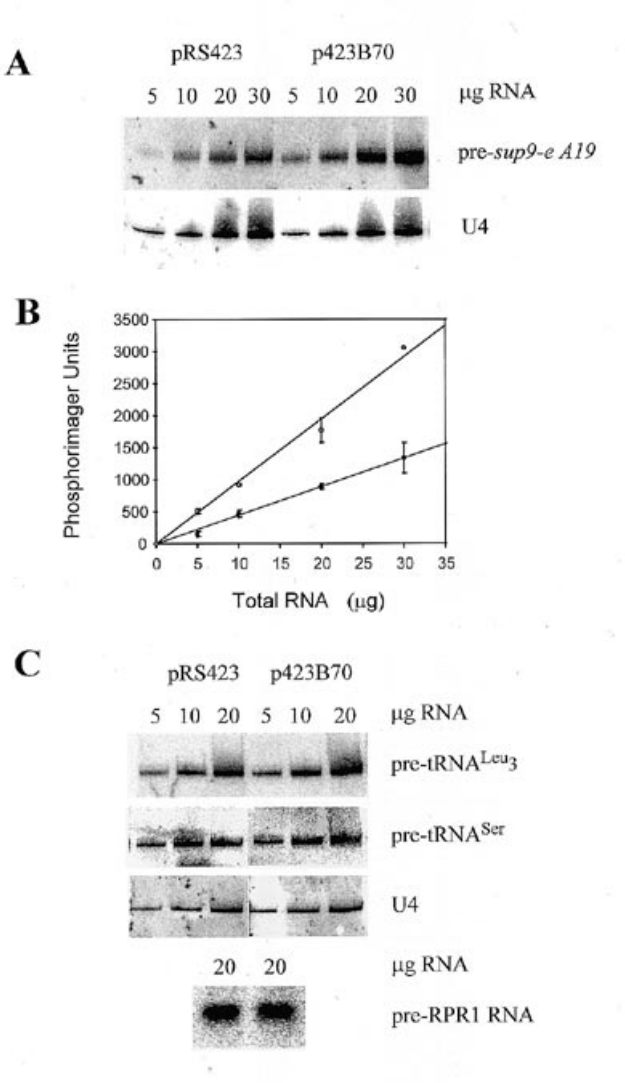

Figure 4. Northern analysis of precursor RNAs transcribed by pol III. (**A**) Total RNA (5, 10, 20 and 30 μg) from a wild-type (pRS423) and a TFIIIB$_{70}$ overexpression (p423B70) strain was analyzed for 5′ pre-*sup9-e A19* RNA (108 nt precursor) or U4 RNA as indicated. (**B**) Phosphorimage analysis of 5′ pre-*sup9-e A19* RNA from wild-type (●) and TFIIIB$_{70}$ overexpressing (○) strains. RNA values were normalized to those for U4 RNA. The slopes determined by linear regression were used to normalize the data from two independent blots and to derive the standard deviations. (**C**) Total RNA from a wild-type (pRS423) and a TFIIIB$_{70}$ overexpression (p423B70) strain was detected for pre-tRNA$^{Leu}_3$ (CAG), pre-tRNA^{Ser} (CGA), U4 RNA and pre-RPR1 RNA (the precursor of yeast RNase P RNA). The data were analyzed as described above and in Materials and Methods.

increase in nonsense suppressor activity. In support of this idea, other studies have observed that tRNA steady state levels remain constant despite changing levels of tRNA synthesis (2,39). In order to measure the relative levels of synthesis of pol III transcripts *in vivo*, northern analysis was performed using probes for short-lived precursor RNAs. Previous studies have shown that an intron-specific pre-tRNA probe provides a reliable measure of RNA synthesis by pol III (40,41). Since the preferred order of processing for pre-tRNAs proceeds with 5′ end trimming, 3′ end trimming and then intron splicing (35), we chose to use 5′ pre-tRNA probes to detect the shortest-lived tRNA precursors. Total RNA was extracted from wild-type and TFIIIB$_{70}$ overexpressing strains and subjected to northern analysis. The blots were hybridized with a series of 5′ pre-tRNA-specific oligonucleotides and other probes including one for the pol II-transcribed U4 snRNA which served as an internal control. Figure 4A shows a representative northern blot indicating the levels of 5′ pre-*sup9-e A19* RNA. The quantitation of this blot together with that from a second independent experiment revealed a 2-fold increase in the level of the 5′ flanked tRNA precursor (Fig. 4B). This result is in precise agreement with the quantitative nonsense suppression assay (Table 1). Accordingly, the 2-fold increase in the synthesis of 5′ pre-*sup9-e A19* RNA most likely underlies the corresponding increase in *supS1* amber suppressor activity. Together with the other experiments presented thus far, this result also indicates that the 2-fold *in vivo* effect and the 17-fold *in vitro* effect of overexpressing TFIIIB$_{70}$ represent true quantitative differences in the synthesis of *sup9-e A19-supS1* RNA.

Surprisingly, overexpression of TFIIIB$_{70}$ did not increase the level of other pol III-transcribed precursor RNAs (Fig. 4C), specifically pre-tRNA$^{Leu}_3$ (CAA), pre-tRNA^{Ser} (CGA) and pre-RPR1 (the RNA component of yeast RNase P). Similarly, several mature pol III transcripts showed no difference in amount between the two strains (data not shown). The fact that the mutant *sup9-e A19* promoter showed an effect of TFIIIB$_{70}$ overexpression *in vivo* (Fig. 4A and B) but various wild-type promoters showed no response (Fig. 4C) suggests that TFIIIB$_{70}$ is present at saturating levels for the transcription of many, if not most pol III genes. However, the factor apparently becomes limiting *in vivo* when promoter strength drops below a critical level.

Wild-type levels of TFIIIB$_{70}$ are not limiting for expression of the monomeric *supS1* suppressor

To further examine the hypothesis that pol III genes bearing normal promoters are not limited in their expression by the wild-type level of TFIIIB$_{70}$, we examined the effect of TFIIIB$_{70}$ overexpression on the activity of the moderately strong monomeric *supS1*amber suppressor. A strain containing an integrated copy of the *supS1* gene was transformed with TFIIIB$_{70}$ and with the other two subunits of TFIIIB, each on a multicopy plasmid. Figure 5 shows the effect of overexpressing these subunits on the monomeric *supS1* suppressor. There appears to be no growth advantage for any of the TFIIIB subunit-overexpressing strains. We conclude that transcription from the wild-type *supS1* promoter *in vivo* is not limited to a significant extent by the amount of any TFIIIB subunit. For TFIIIB$_{70}$, this result contrasts with that observed using the *sup9-e A19-supS1* promoter mutant (Fig. 1) indicating that the increased transcription exhibited upon TFIIIB$_{70}$ overexpression may be restricted to genes with weak or defective promoters.

Determination of the cellular levels of TFIIIB$_{70}$, TBP and TFIIIB$_{90}$

TFIIIB$_{70}$ is clearly limiting in transcription assays conducted using whole cell or partially purified (BRα) extracts (Fig. 2A and refs 32,34) yet the quantitative *in vivo* assays show that TFIIIB$_{70}$ is functionally in excess for all but a defective pol III gene. To explore the basis of this difference, we determined the cellular and extract concentrations of TFIIIB$_{70}$ by quantitative western analysis. We also determined the cellular levels of TBP and TFIIIB$_{90}$ in order to compare the amounts of these factors with

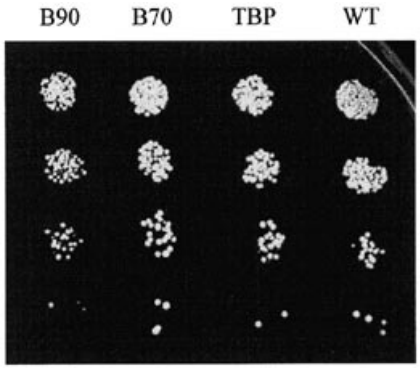

Figure 5. Amber suppressor phenotype of *S.cerevisiae* strain UB7 overexpressing individual TFIIIB Subunits. Strain UB7 which contains an integrated copy of the monomeric *supS1* gene was transformed with the plasmid pRS423 (WT) or derivatives bearing the genes encoding TBP, TFIIIB$_{70}$ (B70) or TFIIIB$_{90}$ (B90). Transformants were grown and plated as described in Figure 1 to monitor *supS1* amber suppressor activity. The cells were photographed after 72 h of incubation at 30°C.

TFIIIB$_{70}$. Figure 6A and B shows a representative western titration of pure recombinant TBP and TFIIIIB$_{70}$ and a global analysis of multiple standard curves for these proteins. The standard curves (Fig. 6B) reveal that the assay is linear over at least a 10-fold range in the amount of loaded protein and that the data are highly reproducible (the error bars are from six individual experiments). Representative blots of TFIIIB$_{70}$ and TBP in a wild-type whole cell lysate are presented in Figure 6C and D together with a quantitation of four independent experiments for each protein. These data and similar experiments for TFIIIB$_{90}$ (not shown) allowed the calculation of absolute numbers of molecules on a per cell basis (Table 2). All three subunits of TFIIIB are present in approximately equal numbers and at levels typical of moderately expressed proteins in yeast (42). The numbers indicate that there is an ~25-fold excess of TFIIIB subunits in each yeast cell relative to the number of pol III genes (43).

Table 2. Quantitation of individual TFIIIB subunits in cells and in extracts

TFIIIB subunit	TBP	TFIIIB$_{70}$	TFIIIB$_{90}$
molecules per cell[a]	0.9×10^4	1.3×10^4	1.0×10^4
nuclear concentration[b]	6.3 µM	8.6 µM	6.6 µM
extract concentration[c]	68 nM	25 nM	nd[d]

[a]Absolute levels per cell represent minimum values since they assume 100% cell breakage and recovery into extract.
[b]2.46 µm^3 was used as the average nuclear volume for an asynchronous cell population (44). The calculation assumes an equal distribution of the proteins throughout the nucleus.
[c]The concentrations are those in a BRα fraction (1.6 mg/ml). This fraction contains ~98% of the TBP, 71% of the TFIIIB$_{70}$ and 10% of the total protein present in the starting crude extract. Thus, the concentrations approximate those found in whole cell extracts at 16 mg/ml.
[d]not determined.

The preceding quantitation of the TFIIIB subunits together with their nuclear localization (Fig. 3 and data not shown) and recent measurements of yeast nuclear volume (44) allows an estimate of their nuclear concentration (Table 2). All three subunits were determined to have concentrations in the low micromolar range. In contrast, the concentrations of TBP and

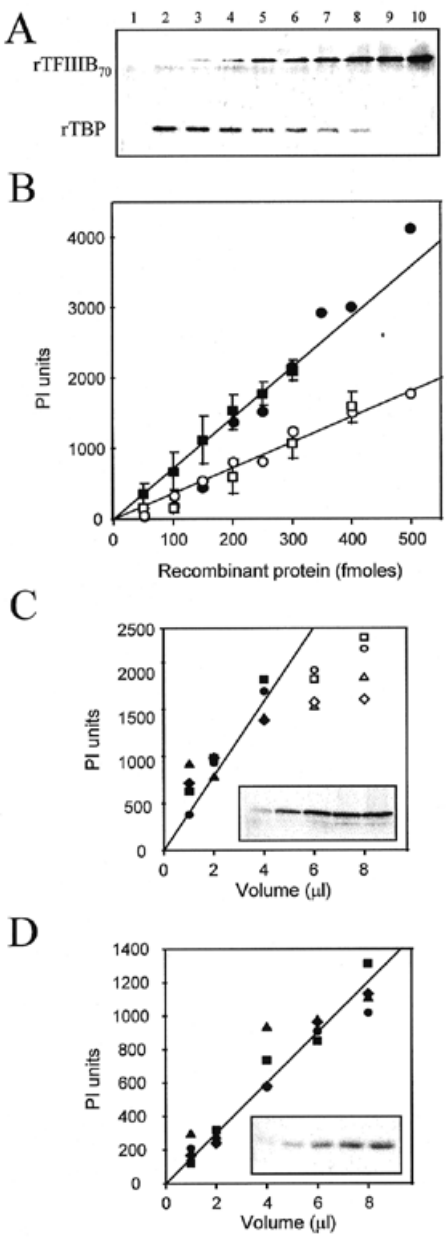

Figure 6. Quantitative western analysis of TFIIIB$_{70}$ and TBP in whole cell lysates. (**A**) Representative western titration of rTFIIIB$_{70}$ (top band) and rTBP (lower band). Lane 1 contains no recombinant proteins, while lanes 2–10 contain 50, 100, 150, 200, 250, 300, 350, 400 and 500 fmol rTFIIIB$_{70}$ and 500, 400, 300, 250, 200, 150, 100, 50 and 0 fmol rTBP, respectively. (**B**) Global linear regression analysis of TFIIIB$_{70}$ (■) and TBP (□) standard curves. Standard deviations are determined for points represented in six independent standard curves. The data from (A), TFIIIB$_{70}$ (●) and TBP (○), are also shown. (**C**) Detection of TFIIIB$_{70}$ in wild-type whole cell lysates. Four independent wild-type whole cell lysates were analyzed to determine the level of TFIIIB$_{70}$. Yeast cells (2.67×10^9) in mid log phase were broken as described in Materials and Methods into 1 ml of RIPA buffer. The inset shows the signal for TFIIIB$_{70}$ on one blot. (**D**) Detection of TBP in wild-type whole cell lysates. The whole cell lysates analyzed in (C) were simultaneously detected with antibodies to TBP. The inset shows the signal for TBP on one blot. The western blots shown in the inset to (C) and (D) were detected in parallel with the standards shown in (A).

TFIIIB$_{70}$ in a partially purified wild-type extract (and in the corresponding crude extract) were 68 and 25 nM, respectively (Table 2). TBP and TFIIIB$_{70}$ are therefore ~100-fold or more

dilute in the extract than they are in the nucleus. *In vitro* transcription assays involve dilutions of these factors from 200- to ~3000-fold relative to their nuclear concentrations. Despite this large dilution, neither TBP nor TFIIIB$_{90}$ is limiting for TFIIIC-dependent gene transcription in whole cell extracts (32,34). On the other hand, the limiting nature of TFIIIB$_{70}$ *in vitro* indicates that its concentration is well below the equilibrium constant for its interaction with the TFIIIC–DNA complex (Fig. 2 and ref. 32). We conclude that the quantitative difference between the *in vivo* and *in vitro* assays regarding the effect of TFIIIB$_{70}$ overexpression (for example, the 2-fold versus the 17-fold effect on the synthesis of *sup9-e A19-supS1*, Figs 2 and 4A) is likely to result in large part from the dilution of TFIIIB$_{70}$ in the extract.

A differential response to TFIIIB$_{70}$ overexpression *in vitro*

Since TFIIIB$_{70}$ overexpression increases transcription of the *sup9-e A19* promoter mutant template but not wild-type templates *in vivo* (Figs 1, 4 and 5), we examined the effect of elevated TFIIIB$_{70}$ levels on the transcription of wild-type and promoter mutant genes *in vitro* to determine if a similar differential response could be observed (Fig. 7). Transcription of the wild-type *sup9-e* gene was increased 11-fold in the YSB108 extract compared with the B6 extract. In contrast, eight promoter mutants including representatives from both the A and B block control elements showed effects ranging from 12- to 23-fold. Interestingly, when the B-block mutant templates were ordered according to their relative transcriptional efficiency in the wild-type extract, an inverse correlation was observed between the fold increase in transcription and promoter strength. The strongest promoter, the wild-type *sup9-e* gene, showed the smallest fold stimulation whereas the weakest promoter, the *sup9-e A53* gene, showed the largest effect. A similar correlation was observed for the A block mutant templates. In addition, when comparing A and B block mutants of similar promoter strength (e.g. compare A19 and T58 or G7 and A55, Fig. 7), the A block mutants showed a larger response to TFIIIB$_{70}$ overexpression. These *in vitro* results correlate with the *in vivo* observations insofar as there is a differential in the transcriptional response of the wild-type and mutant templates to elevated levels of TFIIIB$_{70}$.

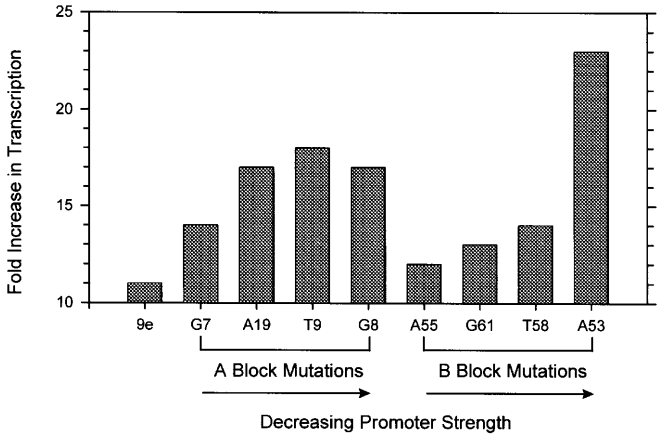

Figure 7. Differential effect of TFIIIB$_{70}$ overexpression on *in vitro* transcription of the dimeric *sup9-e* gene and promoter mutants. The plot shows the effect of overexpressing TFIIIB$_{70}$ on *in vitro* transcription of the wild-type *sup9-e* gene and eight promoter mutants. The mutant templates are grouped by promoter element with transcription activity decreasing from left to right within each element. Transcription activity in the wild-type extract relative to *sup9-e* (1.0) was determined to be 0.47 G7, 0.3 A19, 0.26 T9, 0.19 G8, 0.52 A55, 0.34 G61, 0.29 T58 and 0.09 A53. The fold increase in transcription represents the ratio of the absolute transcription levels in the YSB108 (TFIIIB$_{70}$ overexpressing) and wild-type extracts. All transcription assays were conducted under multiple round conditions (60 min incubations at 15 °C) with 60 µg of extract and 1 µg of template. The results represent the averages from two or three experiments in which the standard deviation from the average fold increase in transcription was <12%.

DISCUSSION

The amount of TFIIIB$_{70}$ in yeast has been shown previously to be limiting for the expression of the promoter mutant *sup9-e A19-supS1* gene *in vivo* (21). However, this initial study did not address whether TFIIIB$_{70}$ was more or less limiting than the other TFIIIB subunits and did not quantify the extent to which these factors are limiting in the cell. In the present work we have investigated these questions using qualitative and quantitative assays to measure the *in vivo* effect of overexpressing the individual TFIIIB subunits. The results demonstrate that with respect to *sup9-e A19-supS1*, TFIIIB$_{70}$ is the principal limiting subunit of TFIIIB. Overexpression of this factor increased *sup9-e A19-supS1* precursor RNA synthesis and suppressor activity 2-fold over control cells whereas TBP and TFIIIB$_{90}$ had a marginal effect or no effect, respectively (Fig. 4, Table 1 and data not shown). This unique ability of TFIIIB$_{70}$ is in good agreement with the finding that multiple copies of TFIIIB$_{70}$ suppress a conditional mutation in the B block-binding subunit of TFIIIC to a much greater extent than either TBP or TFIIIB$_{90}$ (22).

Although the effect of overexpressing TFIIIB$_{70}$ was more pronounced than for the other TFIIIB subunits, overexpression of TBP (10-fold) resulted in a small (1.2-fold) effect on *sup9-e A19-supS1* suppressor activity (Table 1). This result is consistent with the observation that pol II-specific temperature-sensitive mutations in TBP effect small increases in the levels of pol III-specific transcripts (41). Considering that there are somewhat fewer molecules of TBP per cell than either TFIIIB$_{70}$ or TFIIIB$_{90}$ (Table 2) and that TBP is engaged in transcription by all three nuclear RNA polymerases (13) whereas TFIIIB$_{70}$ and TFIIIB$_{90}$ are pol III-specific factors (3,12,22,45), it seems surprising that TBP is not the predominant limiting subunit of TFIIIB. However, the nature of the limiting component is determined by numerous parameters including the cellular concentrations of the factors and the relative affinities of their interactions. In this regard, the genetic data are consistent with biochemical indications that the weakest thermodynamic link in the assembly of the TFIIIB–TFIIIC–DNA complex is the interaction between TFIIIB$_{70}$ and TFIIIC (11,34).

In light of the results obtained with *sup9-e A19-supS1*, we were surprised to find that the expression or synthesis of various wild-type pol III gene products *in vivo* was not limited to a significant extent by the amount of any TFIIIB subunit (Figs 4C and 5, and data not shown). Given the generally close correspondence between tRNA gene promoters and the consensus A and B block elements (46) and correlations indicating that tRNA gene copy number in yeast is the principal determinant of intracellular tRNA levels (47), it seems likely that TFIIIB subunit levels in logarithmically-growing yeast cells are not limiting for the expression of most tRNA genes. This situation appears to change

however as yeast cells exit logarithmic growth and approach stationary phase (32,48). At a relatively early stage in this transition, the amount of TFIIIB$_{70}$ in the cells declines (32). Moreover, the reduction in the cellular level of TFIIIB$_{70}$ at this stage in the growth cycle has been shown to contribute to the reduced transcription observed in extracts from such cells (32). We conclude that TFIIIB$_{70}$ becomes limiting for the transcription of many pol III genes as cell growth rate decreases.

Our results in yeast differ from those obtained in *Drosophila* and rat cell lines where the amount of TBP significantly limits the transcription of pol III genes (23,24). The basis of this difference is not known but could reflect a difference in the availability of TBP for inclusion into TFIIIB complexes. Biochemical studies have shown that TBP in metazoans is found mostly in stable complexes with TATA-binding protein-associated factors (49,50). Yeast cell extracts on the other hand, contain significant amounts of free TBP (19) that (if present *in vivo*) could be sequestered into pol III transcription complexes by increasing the level of TFIIIIB$_{70}$. Alternatively, the limiting nature of TBP for pol III gene transcription in *Drosophila* and vertebrates (51,52) may reflect unique interactions involving the poorly conserved N-terminal domain of TBP (23,53). Deletion of this domain prevents the increase in tRNA and U6 gene transcription that is seen in *Drosophila* cells overexpressing TBP. This indicates an essential role for the N-terminal domain of *Drosophila* TBP in pol III transcription (23). Conversely, the N-terminal domain of yeast TBP does not play a critical role in pol III transcription since its deletion does not affect cell viability or growth rate (54).

The three subunits of TFIIIB are present in yeast cells in approximately equal numbers (Table 2) and are localized to the nucleus (Fig. 3 and data not shown). From these data and volume estimates for yeast nuclei (44), the concentrations of TBP, TFIIIB$_{70}$ and TFIIIB$_{90}$ *in vivo* were determined to be in the low micromolar range. These data have implications for the nature of TFIIIB *in vivo* and for the manner in which it is assembled onto DNA. Recent quantitative studies on the cooperative binding of TBP and TFIIIB$_{70}$ to DNA estimate a dissociation constant for the TBP–TFIIIB$_{70}$ complex of ~120 nM, well below the nuclear concentrations of these factors (Table 2 and Librizzi, Brenowitz and Willis, unpublished data). This implies that TBP and TFIIIB$_{70}$ are complexed with one another *in vivo* in the absence of DNA. Similar quantitative experiments suggest that the binding of TFIIIB$_{90}$ and TBP to DNA is non-cooperative (Librizzi, Brenowitz and Willis, unpublished data) yet the TFIIIB$_{90}$–TBP–DNA complex is stable to electrophoresis in native gels (12). The affinity of the TBP–TFIIIB$_{90}$ interaction is therefore also expected to be in the nanomolar range. Taken together, the data favor the hypothesis that TFIIIB exists as a complex in the nuclei of yeast and suggest that the recruitment of TFIIIB to the promoters of pol III genes is likely to proceed in a single concerted step rather than by the sequential binding of individual TFIIIB subunits. The presence of yeast TFIIIB within even larger complexes, such as the pol III holoenzyme described recently in human cells (55), is also possible (see below).

We determined that the concentrations of TFIIIB$_{70}$ and TBP are >100-fold higher *in vivo* than in typical whole cell extracts. This large dilution of the factors upon extract preparation is likely to affect TFIIIB complex stability *in vitro* and may account for some differences reported in this regard (4,18,19). In a broader context, we note that associations between components of other nuclear complexes may also be affected by the large dilution that occurs with the preparation of extracts. For example, differences concerning the composition of the RNA polymerase II holoenzyme may simply reflect the concentration-dependent stability of some of its components (56). More importantly, the knowledge that nuclear factors are likely to be more concentrated in the cell than in an extract favors their inclusion as subunits of macromolecular complexes.

ACKNOWLEDGEMENTS

We thank Karen Puglia for preparing the IW1B6 and YSB108 whole cell extracts, Roy Long for his expertise and assistance with the immunofluorescence and Shailesh Shenoy and Michael Cammer in the Analytical Imaging Facility at AECOM for producing the immunofluorescence pictures. This work was supported by grants from the NIGMS and the Hirschl-Weill Caulier Trust.

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